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## Identification and isolation of flavonoids from Iraqi *Silybum marianum* L. flowers by HPLC

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**Abstract**---One of the most important phytochemical compounds with potential applications in medicinal chemistry is the flavonoids which exist in many different parts of plants (fruits, herbs, vegetables). Flavonoids possess many medicinal benefits, including antioxidant, antiviral, anticancer, and anti-inflammatory properties. In addition to the cardio and neuro-protective effects. So the present study aimed to identify and isolate flavonoids from the flowers of Iraqi *Silybum marianum* L. by High-Performance Liquid Chromatography-HPLC. The results indicate that the HPLC analysis of plant flavonoids indicate that the flowers contain different concentration of flavonoids which include 119.7143 µg/g of Taxifolin, 307.4991 µg/g of Silychristin A, 137.6423 µg/g of Silidianin, 252.938 µg/g of Silychristin B, 339.9172 µg/g of Silybin A, 378.3294 µg/g of Silybin B, 234.6421 µg/g of Isosilybin A and 127.2572 µg/g of Isosilybin B. The results also indicate that the identification of flavonoids in isolated part are the same in the crude flowers but different concentration, 138.0556 µg/g of Taxifolin, 283.0717 µg/g of Silychristin A, 122.4808 µg/g of Silidianin, 190.1345 µg/g of Silychristin B, 310.9728 µg/g of Silybin A, 363.0253 µg/g of Silybin B, 214.0452 µg/g of Isosilybin A and 126.3279 µg/g of Isosilybin B. So from present of the current study we can conclude that the flowers of *Silybum marianum* are considered a good source of flavonoids with potential health benefits.

**Keywords**---flavonoids, *Silybum marianum*, taxifolin, Silybin A, Silybin B.

## Introduction

Medicinal plants are usually used for a diversity of purposes in herbal medicine, which is based on the premise that medicinal plants contain natural chemical compounds that can alleviate illness or promote health[1]. This effect is mainly due to the presence of secondary metabolites (such as Phenolic compounds, flavonoids, sterols, terpenoids, alkaloids, saponins... etc)[2,3]. These metabolites have some antioxidants properties, especially phenolic compounds and flavonoids[4].

Flavonoids are a category of natural substances belonging to the family of polyphenols, which play a vital role in photosynthesizing cells[5]. Generally, they have the structure of 15-carbon atoms arranged in three rings, consisting of two phenyl rings(A and B) and a heterocyclic pyran ring (C), as shown in Fig.1[6].

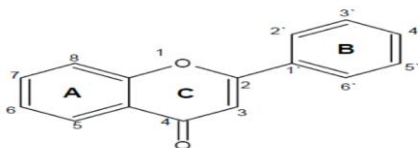


Fig.1: Chemical Structure of the Basic Flavonoid Skeleton<sup>(6)</sup>.

The differences in this basic chemical structure bear the several subclasses of flavonoids compounds. These are flavones, isoflavones, flavonols, chalcones, flavanones, flavanols (catechins) and anthocyanins [7]. Flavonoids are found in vegetables and fruits, and prevent the body from different diseases such as cancer[8], Alzheimer's disease[9], atherosclerosis[10], COVID-19[11]...etc. there are linked with a wide spectrum of health-promoting effects and are an essential component in a set of cosmetic, medicinal, nutraceutical, and pharmaceutical applications. This is because of their anti-carcinogenic, antioxidant, anti-mutagenic, and anti-inflammatory properties and their ability to improve key cellular enzyme physiological functions[7, 12].

*Silybum marianum* L.(also known as milk thistle) of the Asteraceae family, is a yearly/biennial plant cultivated and growing all over the world[13].The results of many experimental studies indicated that the plant has many pharmaceutical and biological properties such as antioxidant, anticancer, Anti-Aging, anti-inflammatory, anti-fungal, and antibacterial activities and also has a hepatoprotective effect and protective effect against DNA injury[14-16]. The biological and pharmaceutical effect of the plant may be due to the chemical constituents which include fatty acids, phenolic compounds, flavonoids (such as quercetin, naringin, kaempferol, chrysoeriol, apigenin, taxifolin), flavonolignans (such as silymarin, silychristin, silydianin) sterols (such as sitosterol, cholesterol, stigmasterol, cholesterol ) and also contain proteins and monosaccharides rhamnose, glucose, arabinose and xylose)[17,18]. So the

present study aimed to identification and isolation of flavonoids from Iraqi *Silybum marianum L.* flowers by High Performance Liquid Chromatography-HPLC.

### Material and methods:

- Plant material:- the dry plant of *Silybum marianum L.* was obtained from the traditional market in Balad city, Salah El-Dean, Iraq. The flowers were separated from other parts of plant and made a fine powder, and kept in a dark container until used.
- Solvents used: All solvents used in the extraction of flavonoids and HPLC analysis were HPLC grade.
- Extraction of flavonoids: The extraction of flavonoids from *Silybum marianum L.* flowers-SMF was done according to the method of [19] with some modification. In which 100gm of SMF powder was extracted with 500ml petroleum ether was used before extraction to remove using soxhlet apparatus for 3 hours to remove fatty contents, and then 500ml of 80% ethanol was used instead of 70% in original method.
- Identification of flavonoids by HPLC: Identification and quantitative determination of flavonoids in plant flowers and its isolated flavonoids were carried out according to [20] method. In which phenomenex C-18 column(50X2.0 mmID) was used, while the mobile phase used was:
  - solvent A(5% formic acid in water)
  - solvent B ( 5%formic acid in methanol), a linear gradient of solvents was used as (0% B to 100% B for 20 min) with flow rate=1.4ml/min, the detection was done at UV 370nm.
- Sample preparation for HPLC: 10gm of plant flower or isolated flavonoid were homogenized in polyton in an ice bath with 20ml of ( 4mM sodium fluoride in methanol to inactivate polyphenol oxidases and prevent phenolic degradation due to browning) [21], centrifuged in a cooling centrifuge at 2-5 °C for 15min with speed (16000g ). The supernatant was filtered through 0.45mm micro-filters and direct injection in the HPLC column for analysis.
- Standard flavonoids: Twenty five µg/ml of eight standard flavonoids were used, which include(Taxifolin, Silychristin A, Silidianin, Silychristin B, Silybin A, Silybin B, Isosilybin A and Isosilybin B).The concentration of identified flavonoids in plant flower and in isolated flavonoids were done according to the following equation:

$$\frac{\text{Area of sample}}{\text{Area of standard}} \times 25 \times D$$

### Results and Discussion

The current study includes the identification of some types of flavonoids in Iraqi *Silybum marianum L.* flowers by HPLC. Eight types of standard flavonoids were used Taxifolin, Silychristin A, Silidianin, Silychristin B, Silybin A, Silybin B, Isosilybin A and Isosilybin B. Fig 2 shows the peaks of the eight standards, and the retention time-Rt and area under curves were showed in table 1.

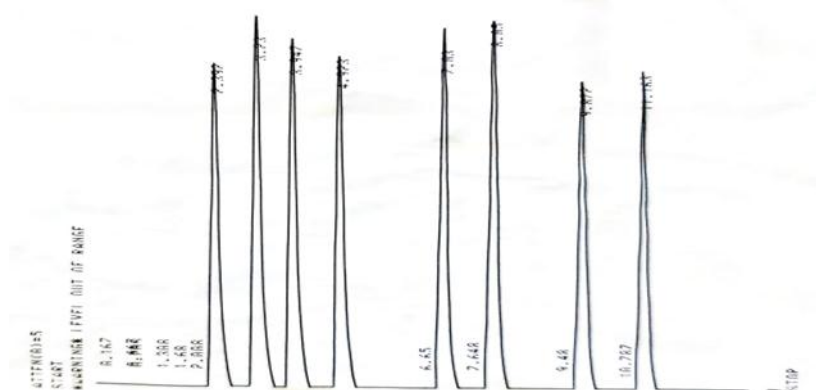


Fig. 2: The peaks of standard flavonoids

Table 1: The Rt and area under curve of standard flavonoids

| Standard flavonoids | Retention time(minute) | Area under curve ( $\mu$ volt) |
|---------------------|------------------------|--------------------------------|
| Taxifolin           | 3.392                  | 390169                         |
| Silychristin A      | 3.23                   | 443771                         |
| Silidianin          | 3.947                  | 430407                         |
| Silychristin B      | 4.923                  | 395338                         |
| Silybin A           | 7.03                   | 436271                         |
| Silybin B           | 8.05                   | 469521                         |
| Isosilybin A        | 9.872                  | 387263                         |
| Isosilybin B        | 11.163                 | 388698                         |

The HPLC analysis of the plant's flowers and its isolated flavonoids were shown in Fig 3 and 4 respectively.

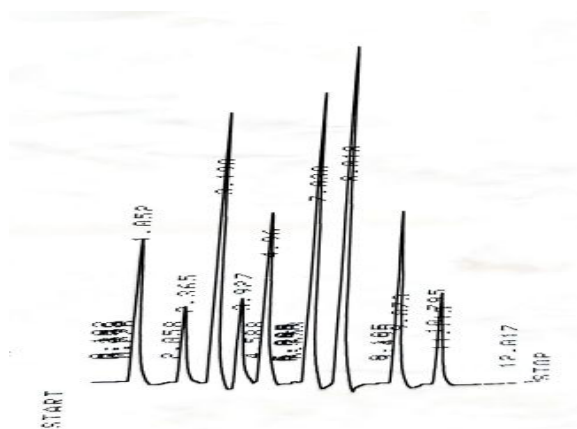


Fig.3:HPLC analysis of flavonoids in *Silybum marianum* L. flowers

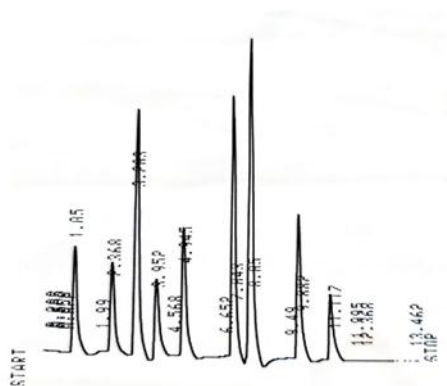


Fig.4:HPLC analysis of flavonoids in isolated flavonoids

According to the Rt of each peak in table 1, the types of flavonoids were identified in *Silybum marianum* flowers and their isolated flavonoids. The results indicate that the flowers of the plant and their isolated flavonoids contain eight types of flavonoids with different concentration, Table 2, and Table 3 respectively.

Table 2:The retention time and area under curve of Iraqi *Silybum marianum* flowers flavonoids by HPLC analysis

| Rt (min) | Area under curve (μvolt) | Identified compounds | Concentration μg/g |
|----------|--------------------------|----------------------|--------------------|
| 1.052    | 118267                   | unknown              | -----              |
| 2.058    | 5869                     | unknown              | -----              |
| 2.365    | 77848                    | Taxifolin            | 119.7143           |
| 3.198    | 227432                   | Silychristin A       | 307.4991           |
| 3.927    | 98737                    | Silidianin           | 137.6423           |
| 4.96     | 166660                   | Silychristin B       | 252.938            |
| 5.895    | 9987                     | unknown              | -----              |
| 6.082    | 8399                     | unknown              | -----              |
| 6.265    | 2792                     | unknown              | -----              |
| 6.398    | 4285                     | unknown              | -----              |
| 6.653    | 13630                    | unknown              | -----              |
| 7.038    | 247160                   | Silybin A            | 339.9172           |
| 8.018    | 296056                   | Silybin B            | 378.3294           |
| 9.105    | 18462                    | unknown              | -----              |
| 9.495    | 13437                    | unknown              | -----              |
| 9.873    | 151447                   | Isosilybin A         | 234.6421           |
| 10.795   | 6105                     | unknown              | -----              |
| 11.115   | 82441                    | Isosilybin B         | 127.2572           |

Table 3:The retention time and area under curve of isolated flavonoids from Iraqi *Silybum marianum* flowers flavonoids by HPLC analysis

| Rt (min) | Area under curve (μvolt) | Identified compounds | Concentration μg/g |
|----------|--------------------------|----------------------|--------------------|
|----------|--------------------------|----------------------|--------------------|

|        |        |                |          |
|--------|--------|----------------|----------|
| 1.05   | 89169  | unknown        | -----    |
| 1.99   | 8900   | unknown        | -----    |
| 2.365  | 89775  | Taxifolin      | 138.0556 |
| 3.203  | 209365 | Silychristin A | 283.0717 |
| 3.952  | 87861  | Silidianin     | 122.4808 |
| 4.945  | 125279 | Silychristin B | 190.1345 |
| 6.652  | 39044  | unknown        | -----    |
| 7.043  | 226114 | silybin A      | 310.9728 |
| 8.05   | 284080 | silybin B      | 363.0253 |
| 9.49   | 31166  | unknown        | -----    |
| 9.882  | 138153 | Isosilybin A   | 214.0452 |
| 11.117 | 81839  | Isosilybin B   | 126.3279 |
| 11.995 | 6486   | Unknown        | -----    |
| 12.368 | 5684   | unknown        |          |

The results indicate that the flowers of *Silybum marianum* contain different concentrations of flavonoids, which include Taxifolin, Silychristin A, Silidianin, Silychristin B, Silybin A, Silybin B, Isosilybin A, and Isosilybin B with ten unknown peaks. In an H<sub>2</sub>O:MeOH extract of plant flowers Ahmed, *et al*[22], identified seven types of flavonoids: kaempferol, apigenin 7-O- $\beta$ -galactoside, apigenin 7-O- $\beta$ -(2''-O- $\alpha$ -rhamnosyl)galacturonide, apigenin 7-O- $\beta$ -glucoside, kaempferol 3-O- $\alpha$ -rhamnoside-7-O- $\beta$ -galacturonide, kaempferol-3-O- $\alpha$ -rhamnoside and apigenin 7-O- $\beta$ -glucuronide 6''-ethyl ester. Lv, *et al*[23] indicate that the biosynthesis of taxifolin exists in the flower, then it is transmitted to the pericarp where the synthesis of silymarin constituents occurs. While Martin, *et al*[24] identified some types of flavonoids in the plant flowers which include silychristin A, silychristin B, silybin A, silybin B, silydianin, small amount of silymarin and Isosilybin B.

The study also include isolation the flavonoids from *Silybum marianum* flowers, and identified the type of flavonoids in isolated extract by HPLC. The result indicate the same flavonoids that identified in the crude flowers, with six unknown peaks. No information was available in the literatures about isolation of flavonoids from plant flowers or about the types of flavonoids in isolated flavonoids, so the present study is a novel study that isolated the flavonoids from *Silybum marianum* flowers and characterize novel flavonoids in the isolated extract.

In the last twenty years, many researchers become interested to study the pharmacological activities of flavonoids and use different techniques to isolate and identify them, intending to evaluate their potential health benefits[25]. The presence of flavonoids in the diet help in the prevention of some diseases such as cardiovascular disease, cancer, inflammation, diabetes mellitus... etc[26,27], and also the isolated flavonoids from different plants showed pharmacological properties, In which the isolated flavonoids from *Arctium Lappa* Stem showed hepatoprotective effect[28], and also the isolated flavonoids from Iraqi date palm pollen showed hypolipidemic and anti-atherosclerotic effects[29]. So more studies were required to identify the pharmacological effect of isolated flavonoids from *Silybum marianum* flowers.

The biological activities of flavonoids rely on the type of identified flavonoid, its bioavailability, and its potential mode of action. So many researchers study the biological activities of different types of flavonoids, Wang, et al[30,31] showed that taxifolin can modulate NF- $\kappa$ B signaling pathway thus improving oxidative damage, in addition to different pharmacological effects, including anti-inflammatory[32], anti-cancer[33], antioxidant[34], neuroprotective activities[35] and anti-inflammatory[36]. While Silychristin A showed antioxidant anti-inflammatory activities[37], also silicristin and silidianin exhibited anticancer and antioxidant activity[38]. Thus the presence of these compounds in plants considers a primary predictor for plant potential activity.

## Conclusion

From all the above results we can conclude that the flowers of *Silybum marianum* are considered a good source of flavonoids with potential health benefits.

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